ELISpot

Mouse $CD4^+$/IFN-γ

Catalog Number EL2019

For the quantitative determination of the frequency of $CD4^+$ cells releasing mouse IFN-γ.

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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INTRODUCTION

Mouse interferon-gamma (IFN-γ) is a 37 kDa, non-disulfide linked homodimeric glycoprotein that plays a key role in cell-based immunity (1, 2). Synthesized as a 156 amino acid (aa) precursor, the mature molecule is 136 amino acids in length and takes the shape of a 4 α-helix bundle with N- and C-terminal extensions (2, 3). As with human IFN-γ, mouse IFN-γ is known to undergo some C-terminal proteolytic processing (2, 4, 5). At the N-terminus, human IFN-γ shows cleavage of the first three amino acids (C-Y-C) and conversion of the subsequent glutamine to pyroglutamate (5). In mouse, while the first three N-terminal amino acids are identical to human, the fourth aa is histidine, and it is not certain how much processing occurs in this region. Functionally, the N-terminus is associated with receptor binding (3, 6), while the C-terminus holds sequence motifs for various functions including nuclear localization (7), heparin-binding (8), and receptor interaction (3). Relative to human IFN-γ, mouse IFN-γ is 10 amino acids shorter in the mature segment, with the bulk of amino acids missing at the extreme carboxy-terminus (2). Mouse to human, there is only 40% aa sequence identity, and this is reflected in the fact that mouse IFN-γ is inactive on human cells. Relative to rat IFN-γ, mouse IFN-γ is 87% aa identical and likely to be active on rat cells since rat IFN-γ is active on mouse cells (9). Mouse cells known to express IFN-γ include B cells (10), dendritic γδ T cells (11), $CD4^+$ and $CD8^+$ T cells (12), $CD8\alpha^+$ lymphoid dendritic cells (13), NK cells plus NK 1.1^+ T cells (14), and macrophages (13, 15). In rats, IFN-γ production has been reported in mast cells (16) and sensory neurons (17).

The receptor for mouse IFN-γ consists of two components, a 90 kDa ligand-binding α-chain (IFN-γ R1) (18 - 21), and a 63 kDa signal-transducing β-chain (IFN-γ R2) (18, 22). The α-chain is a type I transmembrane glycoprotein that is 451 amino acids in length, with a 228 aa extracellular region and a 200 aa cytoplasmic domain that is marked by an extraordinarily high percentage of Serine/Threonine residues (25%). Mouse to human, the IFN-γ R α-chain is 52% aa identical. The β-chain of the receptor is also a type I transmembrane glycoprotein that is 314 aa in length and contains a 224 aa extracellular region and a 66 aa cytoplasmic domain. A comparison of mouse and human receptor β-chains show 58% aa identity (18, 22). The Kd for mouse IFN-γ binding to the mouse IFN-γ R lies between 50 - 500 pM (23). The functional receptor/ligand complex is thought to consist of a homodimeric IFN-γ plus two α- and two β-chains (18).

Functionally, IFN-γ is suggested to play a number of roles in cellular immunity. In particular, it inhibits Th2 differentiation and, in the presence of IL-12, stimulates Th1 development. It also activates macrophages by 1) upregulating reactive oxygen and NO levels, 2) inducing membrane FcγRI and III expression (for phagocytosis and ADCC), and 3) promoting the secretion of TNF-α, IL-1β, IL-10, MIG, and IP-10. On B cells, IFN-γ induces IgG isotype secretion and downregulates IgE production, while on endothelial cells, IFN-γ upregulates the expression of ICAM-1 and E-selectin (24, 25). In total, IFN-γ induces immune responses that fight parasites and intracellular bacteria. In the anti-viral arena, IFN-γ apparently performs at least two functions. First, it induces enzymes which interfere with normal RNA function. This results in either non-functional protein translation or an inhibition of protein synthesis (25). Second, it modulates the level of both TRAIL and TRAIL R on virally-infected cells, thus inducing their death (26). Molecules known to induce IFN-γ include IL-12 and IL-18 (27 - 29).

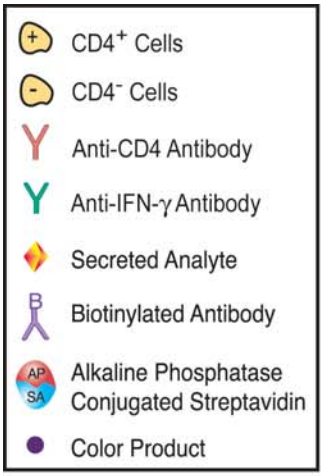
Mouse CD4 is a 55 kDa type I transmembrane palmitoylated glycoptotein that contains four extracellular Ig-like domains (30, 31). It binds to MHC-II molecules, and acts as a co-receptor for the T cell receptor (TCR). This facilitates TCR signaling, and either expands the repertoire of antigens that the TCR recognizes or increases the avidity of TCR for antigen (32, 33). It also binds IL-16, inducing T cell migration (34). In the extracellular region, CD4 shows 72%, 53% and 46% aa sequence identity to rat, human, and canine CD4, respectively (30).

The Mouse $CD4^+$/IFN-γ ELISpot assay is designed for the detection of IFN-γ secreted from $CD4^+$ cells at the individual single cell level and can be used to quantitate the frequency of mouse IFN-γ secreting cells. ELISpot assays are well suited for studying immune responses to various treatments and therapies and have been used for the quantitation of antigen-specific $CD4^+$ and/or $CD8^+$ T cell responses. Other methods for assessment of antigen-specific T cell responses, such as chromium release assay with quantitation by limiting dilution are tedious and require previous *in vitro* expansion of T cells for several days. These assays typically are not suitable for measuring infrequent T cell responses that occur at less than 1 in 1000. ELISpot assays are highly reproducible and sensitive and can be used to measure responses with frequencies well below 1 in 100,000. ELISpot assays do not require prior *in vitro* expansion of T cells and are suitable for high-throughput analysis using only small volumes of primary cells. As such, ELISpot assays are useful tools for research in vaccine development and for the monitoring of various clinical trials.

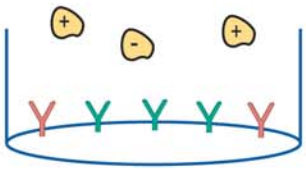
PRINCIPLE OF THE ASSAY

The enzyme-linked immunospot (ELISpot) assay was originally developed for the detection of individual B cells secreting antigen-specific antibodies (35, 36). This method has since been adapted for the detection of a selected cell type secreting specific cytokines or other antigens (37, 38). ELISpot assays employ the quantitative sandwich enzyme-linked immunosorbent assay (ELISA) technique. PVDF (polyvinylidene difluoride)-backed microplates are coated with both anti-mouse CD4 and anti-mouse IFN-γ monoclonal antibodies. Cells are added to the plate and incubated for a short period of time to capture $CD4^+$ cells. After that, plates are washed to remove unbound non-$CD4^+$ cells. The ELISpot plate with $CD4^+$-enriched cells is placed into a 37° C CO_2 incubator for a specifies period of time. During the incubation, anti-mouse IFN-γ antibodies capture IFN-γ screted primarily by $CD4^+$ cells. After washing away any cells and unbound substances, a biotinylated polyclonal antibody specific for mouse IFN-γ is added to the wells. Following a wash to remove any unbound biotinylated antibody, alkaline-phosphatase conjugated to streptavidin is added. Unbound enzyme is subsequently removed by washing and a substrate solution (BCIP/NBT) is added. A blue-black colored precipitate forms at the sites of cytokine localization and appear as spots, with each individual spot representing an individual IFN-γ secreting cell. The spots can be counted with an automated ELISpot reader system or manually using a stereomicroscope.

ELISpot SCHEMATIC

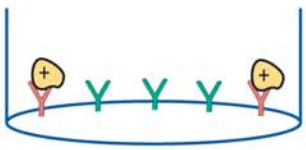


1.



Incubate cell solution for 1 hour at room temperature and then wash.

2.



$CD4^+$ cells are bound by $CD4^+$ capture antibodies. These cells can then be stimulated to release IFN-γ.

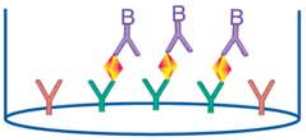
3.

Incubate IFN-γ-secreting cells in an antibody coated well.

4.

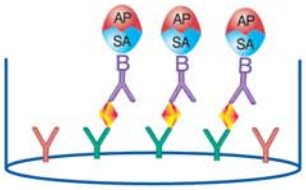
Secreted IFN-γ is captured by the immobilized IFN-γ capture antibody.

5.



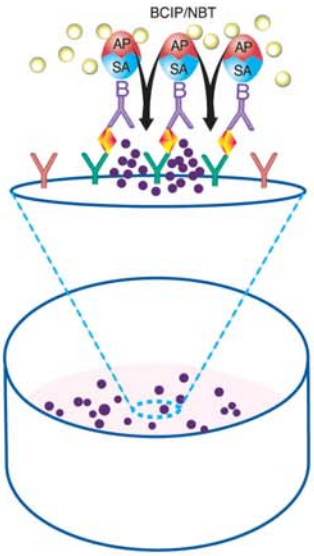
Incubate with biotinylated anti-IFN-γ antibody.

6.



Incubate with alkaline phosphatase conjugated streptavidin.

7.



Add substrate and monitor the formation of colored spots.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- The kit should not be used beyond the expiration date on the kit label.
- Do not mix or substitute reagents with those from other lots or sources.
- Any variation in pipetting and washing techniques, incubation time or temperature, and kit age can cause variation in density of spots, intensity of specific staining and background level.

PRECAUTIONS

Some components of this kit contain sodium azide, which may react with lead and copper plumbing to form explosive metallic azides. Flush with large volumes of water during disposal.

Do not remove the flexible plastic underdrain on the bottom of the microplate before or during incubation and development since it may damage the PVDF membrane filters. The underdrain cover may be removed only after completing the incubation with BCIP/NBT chromogen.

Although the toxicity of the chromogenic substrate BCIP/NBT is not currently known, wear gloves to avoid contact with skin. Follow local, state and federal regulations to dispose of used BCIP/NBT.

MATERIALS PROVIDED

Mouse $CD4^+$/IFN-γ Microplate (Part 892870) - One 96-well PVDF-backed microplate coated with anti-mouse $CD4^+$ and anti-mouse IFN-γ monoclonal antibodies.

Detection Antibody Concentrate (Part 892871) - 150 µL of a 120X concentrated solution of biotinylated polyclonal antibody specific for mouse IFN-γ with preservatives.

Streptavidin-AP Concentrate A (Part 895358) - 150 µL of a 120X concentrated solution of Streptavidin conjugated to Alkaline Phosphatase with preservatives.

Dilution Buffer 1 (Part 895307) - 12 mL of a buffer for diluting Detection Antibody Concentrate with preservatives.

Dilution Buffer 2 (Part 895354) - 12 mL of a buffer for diluting Streptavidin-AP Concentrate A with preservatives.

Wash Buffer Concentrate (Part 895308) - 50 mL of a 10X concentrated solution of a buffered surfactant with preservatives.

BCIP/NBT Chromogen (Part 895867) - 12 mL of a stabilized mixture of 5-Bromo-4-Chloro-3′ Indolylphosphate p-Toluidine Salt (BCIP) and Nitro Blue Tetrazolium Chloride (NBT).

Mouse $CD4^+$/IFN-γ Positive Control (Part 892872) - 1 vial (8 ng) of recombinant mouse IFN-γ; lyophilized.

STORAGE

Store the unopened kit at 2 - 8° C. Do not use beyond the kit expiration date. This kit is validated for single use only. Results obtained with opened/reconstituted reagents at a later date may not be reliable.

OTHER SUPPLIES REQUIRED

- Pipettes and pipette tips
- Deionized or distilled water
- Dulbecco's phosphate-buffered saline (PBS)
- Squirt bottle, manifold dispenser, or automated microplate washer
- 500 mL graduated cylinder
- 37° C CO_2 incubator
- Sterile culture media
- Dissection microscope or an automated ELISpot reader

TECHNICAL HINTS

- To minimize edge effect, place the microplate (bottom down) onto a piece of aluminum foil (about 4 x 6 inches). Add cells, cover the microplate with the lid and shape the foil around the edges of the microplate. The foil may be left on the microplate for the rest of the experimental procedure and removed after the BCIP/NBT has been washed off.
- Do not touch PVDF membrane filters with pipette tips when pipetting cells and reagents to avoid damage to the membrane.
- After completion of the experiment, do not dry the microplate at a temperature higher than 37° C since it may cause cracking of the PVDF membrane filters.
- The 96-well microplate provided in the kit is not sterile. However, due to the short incubation period and the presence of antibiotics in the culture media, microbial contamination has not been a problem during the ELISpot procedure.
- The kit is designed for single use only. The layout of the assay should be carefully planned to maximize the use of the plate and reagents provided.
- The controls listed are recommended for each ELISpot experiment.

  Positive Control - Use recombinant mouse IFN-γ.

  Unstimulated/Negative Control - Use the same number of unstimulated cells as stimulated cells.

  Background Control - Use sterile culture media.

  Detection Antibody Control - Substitute phosphate buffered saline for Detection Antibody.

REAGENT PREPARATION

Wash Buffer - If crystals have formed in the concentrate, warm to room temperature and mix gently until the crystals have completely dissolved. To prepare Wash Buffer, add 50 mL of Wash Buffer Concentrate to 450 mL of deionized water and mix well.

Mouse $CD4^+$/IFN-γ Positive Control - Reconstitute lyophilized mouse IFN-γ with 250 µL of culture medium that is used to incubate cells.

Detection Antibody - Tap or vortex the vial to release reagent collected in the cap. Transfer 100 µL of Detection Antibody Concentrate into the vial labeled Dilution Buffer 1 and mix well. **For optimal performance, prepare Detection Antibody immediately before use.**

Streptavidin-AP - Tap or vortex the vial to release reagent collected in the cap. Transfer 100 µL of Streptavidin-AP Concentrate A into the vial labeled Dilution Buffer 2 and mix well. **For optimal performance, prepare Streptavidin-AP immediately before use.**

SAMPLE PREPARATION

The types of effector and responder cells used, method of cell separation, mode of stimulation, and length of incubation are to be determined by each investigator. R&D Systems' cell selection products are suitable for the purification of effector and responder cells. For a complete product listing of human, mouse, and rat cell selection products, see the R&D Systems catalog or visit our website at www.RnDSystems.com.

ASSAY PROCEDURE

Bring all reagents as needed to room temperature, except the Detection Antibody Concentrate and Dilution Buffer 1, which should remain at 2 - 8° C. All samples and controls should be assayed at least in duplicate. An Assay Record Template is provided at the back of this insert to record controls and samples assayed.

1. Fill all wells in the microplate with 200 µL of sterile culture media and incubate for approximately 20 minutes at room temperature.
2. When cells are ready to be plated, aspirate the culture media from the wells. Immediately add 100 µL of the appropriate cells to each well.
3. Incubate cells for 1 hour at room temperature on the benchtop.
4. Wash away cells which have not been captured by washing with PBS. Dump the contents into the sink and then fill with PBS and repeat two more times. **Do not aspirate cells.**
5. Add 100 µL of culture media containing the appropriate stimulant concentration and 100 µL of the appropriate controls (see Technical Hints for appropriate controls).
6. Incubate cells in a humidified 37° C CO_2 incubator. Optimal incubation time for each stimulus should be determined by the investigator. **Do not disturb the cells during the incubation period.**
7. Aspirate each well and wash, repeating the process three times for a total of four washes. Wash by filling each well with Wash Buffer (250 - 300 µL) using a squirt bottle, manifold dispenser or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
   Note: Adjust the height of the prongs of the manifold dispenser or autowasher to prevent damage to the membranes.
8. Add 100 µL of diluted Detection Antibody into each well and incubate at 2 - 8° C overnight.
9. Repeat step 7.
10. Add 100 µL of diluted Streptavidin-AP into each well and incubate for 2 hours at room temperature.
11. Repeat step 47.
12. Add 100 µL of BCIP/NBT Chromogen into each well and incubate for 1 hour at room temperature. **Protect from light**.
13. Discard the chromogen solution from the microplate and rinse the microplate with deionized water. Invert the microplate and tap to remove excess water. Remove the flexible plastic underdrain from the bottom of the microplate, wipe the bottom of the plate thoroughly with paper towels and dry completely either at room temperature (60 - 90 minutes) or 37° C (15 - 30 minutes).

CALCULATION OF RESULTS

The developed microplate can be analyzed by counting spots either manually using a dissection microscope or by using a specialized automated ELISpot reader. Specific spots are round and have a dark center with slightly fuzzy edges. Quantitation of results can be done, for example, by calculating the number of spot forming cells (SFC) per number of cells added into the well.

REPRODUCIBILITY DATA

Splenocytes from a C57BL mouse (5 x 10^6 cells/mL) were incubated for 1 hour at room temperature. Following three washes with PBS, stimulants (50 ng/mL of phorbol 12-myristate-13-acetate and 0.5 μg/mL calcium ionomycin) were added to the culture media and incubated overnight at 37° C in a 5% CO_2 incubator. The sample was assayed in eight wells according to the procedure and analyzed with a dissection microscope.

Well	Number of Spots Counted
1	347
2	378
3	395
4	390
5	340
6	355
7	395
8	392

TROUBLESHOOTING GUIDE

Observation	Problem	Corrective Action
Following the incubation with BCIP/NBT chromogen and rinsing the microplate with deionized water, the dark-blue background color of filter membrane attenuates visualization and quantitation of spots.	Wet membrane	Microplates cannot be analyzed accurately until PVDF filter membranes are completely dry. Wait until membrane becomes dry, usually 15 - 30 minutes at 37° C or 60 - 90 minutes at room temperature.
The number of spots in the wells that contained the cells is high but their contrast as well as intensity of staining in the Positive Control wells is low.	Underdevelopment - perhaps as a result of using Streptavidin-AP and/or BCIP/NBT solutions that have not been adjusted to room temperature	Bring the reagents to room temperature before adding to the wells.
The number of spots in the wells that contained cells is lower than expected whereas Positive Control wells turned black-blue.	Cell stimulation problem	Ensure that reagents used to stimulate the cytokine release from the cells retained their biological activity. One way to check is to perform immunocytochemistry on fixed cells after stimulation.
	Too few cells added to the wells	Increase the number of cells added per well.
Following incubation with BCIP/NBT and drying the microplate, the density of the spots makes it difficult to quantify them.	Too many cells were added to the wells	Make dilutions of cells (*i.e.,* 1×10^6, 5×10^5, 1×10^5, 5×10^4, 1×10^4 cells per well) to determine the optimal number of cells that will result in formation of distinct spots.

REFERENCES

1. Billiau, A. (1996) Adv. Immunol. **62**:61.
2. Gray, P.W. and D.V. Goeddel (1983) Proc. Natl. Acad. Sci. USA **80**:5842.
3. Jarpe, M.A. and H.M. Johnson (1990) J. Immunol. **145**:3304.
4. Curling, E.M.A. *et al*. (1990) Biochem. J. **272**:333.
5. Rinderknecht, E. *et al*. (1984) J. Biol. Chem. **259**:6790.
6. Magazine, H.I. *et al*. (1988) Proc. Natl. Acad. Sci. USA **85**:1237.
7. Subramaniam, P.S. *et al*. (1999) J. Biol. Chem. **274**:403.
8. Sadir, R. *et al*. (1998) J. Biol. Chem. **273**:10919.
9. Dijkema, R. *et al*. (1985) EMBO J. **4**:761.
10. Yoshimoto, T. *et al*. (1997) Proc. Natl Acad. Sci. USA **94**:3948.
11. Sugaya, M. *et al*. (1999) J. Invest. Dermatol. **113**:350.
12. Hoiden, I. and G. Moller (1996) Scand. J. Immunol. **44**:501.
13. Ohteki, T. *et al*. (1999) J. Exp. Med. **189**:1981.
14. Arase, H. *et al*. (1996) J. Exp. Med. **183**:2391.
15. Puddu, P. *et al*. (1997) J. Immunol. **159**:3490.
16. Gupta, A.A. *et al*. (1996) J. Immunol. **157**:2123.
17. Neumann, H. *et al*. (1997) J. Exp. Med. **186**:2023.
18. Bach, E.A. *et al*. (1997) Annu. Rev. Immunol. **15**:563.
19. Gray, P. W. *et al*. (1989) Proc. Natl. Acad. Sci. USA **86**:8497.
20. Munro, S. and T. Maniatis (1989) Proc. Natl. Acad. Sci. USA **866**:9248.
21. Kumar, C.S. *et al*. (1989) J. Biol. Chem. **264**:17939.
22. Hemmi, S. *et al*. (1994) Cell **76**:803.
23. Hemmi, S. *et al*. (1989) Proc. Natl Acad. Sci. USA **86**:9901.
24. Paludan, S.R. (1998) Scand. J. Immunol. **48**:459.
25. Boehm, U. *et al*. (1997) Annu. Rev. Immunol. **15**:749.
26. Sedger, L.M. *et al*. (1999) J. Immunol. **163**:920.
27. Lebel-Binay, S. *et al*. (2000) Eur. Cytokine Netw. **11**:15.
28. Tominaga, K. *et al*. (2000) Int. Immunol. **12**:151.
29. Trinchieri, G. and F. Gerosa (1996) J. Leukoc. Biol. **59**:505.
30. Brady, R.L. and A.N. Barclay (1996) Curr. Top. Microbiol. Immunol. **205**:1.
31. Fragoso, R. *et al*. (2003) J. Immunol. **170**:913.
32. Barclay, A.N. *et al*. (1993) Phil. Trans. R. Soc. Lond. B **342**:7.
33. Bowers, K. *et al*. (1997) Int. J. Biochem. Cell Biol. **29**:871.
34. Lynch, E.A. *et al*. (2003) J. Immunol. **171**:4965.
35. Czerkinsky, C.C. *et al*. (1983) J. Immunol. Methods **65**:109.
36. Sedgwick, J.D. and P.G. Holt (1983) J. Immunol. Methods **57**:301.
37. Czerkinsky, C.C. *et al*. (1984) J. Immunol. Methods **72**:489.
38. Helms, T. *et al*. (2000) J. Immunol. **164**:3723.

ASSAY RECORD TEMPLATE

This template may be used as a record of samples and controls run in an assay.